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Robert M Hagan, Jane Worner-Gibbs, David C Wilton. The interaction of liver fatty acid-binding protein (FABP); with anionic phospholipid vesicles: is there extended phospholipid anchorage under these conditions?. *Biochemical Journal*, 2008, 410 (1), pp.123-129. 10.1042/BJ20071109. hal-00478870

HAL Id: hal-00478870

<https://hal.science/hal-00478870>

Submitted on 30 Apr 2010

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The Interaction of Liver Fatty Acid-binding Protein (FABP) with Anionic Phospholipid Vesicles: Is there extended phospholipid anchorage under these conditions?

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Running Title: Liver FABP Membrane Binding

Abbreviations used: DAUDA, 11-((5-dimethylaminonaphthalene-1-sulphonyl)amino)-undecanoic acid; DOPC, dioleoyl-*sn*-phosphatidylcholine; DOPG, dioleoyl-*sn*-phosphatidylglycerol; Br₄-DSPC, 1,2-distearoyl(9,10-dibromo)-*sn*-phosphatidylcholine; Br₄-DSPG, 1,2-distearoyl(9,10-dibromo)-*sn*-phosphatidylglycerol; lysoPG, lysophosphatidylglycerol; FABP, fatty acid binding protein; SUV, small unilamellar vesicle; TFE, trifluoroethanol; CRABP, cellular retinoic acid binding protein.

ABSTRACT

Liver fatty acid-binding protein (FABP) binds a variety of non-polar anionic ligands including fatty acids, fatty acyl CoAs, lysophospholipids and bile acids. Liver FABP is also able to bind to anionic phospholipid vesicles under conditions of low ionic strength and membrane-binding results in release of bound ligand. However the molecular interactions involved in binding to the phospholipid interface and the mechanism of ligand release are not known. Ligand release could be due to a significant conformational change in the protein at the interface or interaction of a phospholipid molecule with the ligand binding cavity of the protein resulting in ligand displacement. Two portal mutant proteins of liver FABP, L28W and M74W, have now been used to investigate the binding of liver FABP to anionic phospholipid vesicles, monitoring changes in fluorescence and also fluorescence quenching in the presence of brominated lipids. There is a large increase in fluorescence intensity when the L28W mutant protein binds to vesicles prepared from dioleoyl-*sn*-phosphatidylglycerol (DOPG) but a large decrease in fluorescence intensity when the M74W binds to these vesicles. The Br₄ - phospholipid prepared by bromination of DOPG dramatically quenches both L28W and M74W consistent with the close proximity of a fatty acyl chain to the tryptophan residues. The binding of liver FABP to DOPG vesicles is accompanied by only a minimal change in the CD spectrum. Overall the results are consistent with a molecule of anionic phospholipid interacting with the central cavity of the liver FABP possibly involving the phospholipid molecule in an extended conformation.

INTRODUCTION

Liver fatty acid-binding protein (FABP)¹ is a member of a family of structurally related small (14–15 kDa) cytosolic lipid binding proteins that also include intestinal, heart (muscle), adipocyte, ileal, keratinocyte and brain FABP (for recent reviews, see [1-8]). The exact physiological functions of these proteins are unclear, though it is generally thought that they may have a role in the uptake and targeting of fatty acids to various intracellular organelles and metabolic pathways. All further sites of metabolism of long chain fatty acids in the cell involve membrane proteins. In order for a targeting role to operate, the FABP must interact with an intracellular structure such as a membrane interface or receptor/docking protein.

A process involving membrane binding has been advocated for intestinal, muscle and adipose FABP. Fluorescence studies have led to the proposal of a collisional mechanism for FABP-mediated transfer of fatty acids between phospholipid membranes and vesicles (reviewed in [3]). However, such a process was not observed to operate for liver FABP under the same assay conditions and an aqueous phase diffusion mechanism was proposed, not requiring interaction of the protein with a membrane surface [3]. Such assay conditions reflect the physiological condition seen within the cell in terms of ionic strength.

We have previously observed that liver FABP can interact with a phospholipid interface under particular conditions. Thus liver FABP can bind to small anionic phospholipid vesicles, a phenomenon that is enhanced under conditions of low ionic strength (10 mM buffer only) and is consistent with a primary role for non-specific electrostatic interactions between cationic groups on the protein surface and the anionic phospholipid interface under these conditions [9]. Site directed mutagenesis has highlighted the importance of the portal region of the protein in the overall process with the involvement of three cationic residues, Lys-31, Lys-36 and Lys-57 [10]. The interfacial binding phenomenon was initially monitored by loss of fluorescence intensity as the fluorescent fatty acid analogue DAUDA was released from the protein as a result of binding to phospholipid vesicles. This membrane binding must involve a hydrophobic component because whereas initial binding was sensitive to salt concentration, the addition of high salt after binding failed to reverse the binding phenomenon, at least in part [9]. Overall, the binding phenomenon had close parallels to other proteins used as paradigms for binding to anionic vesicles, particularly cytochrome c [11-17] and another structurally-related protein that binds

fatty acids, β -lactoglobulin [18-21]. Such binding assays normally employed low ionic strength conditions to enhance electrostatic interactions.

We have now generated two tryptophan-insertion mutant proteins of liver FABP in proximity to the portal region (L28W and M74W) and one of these mutant proteins, L28W, has proved particularly useful in monitoring ligand binding to liver FABP [22]. The proximity of Trp-28 and Trp-74 to the second fatty acid binding site (site 2) of liver FABP is shown in Figure 1. In this paper we have used the L28W and M74W mutant proteins to demonstrate binding to phospholipid vesicles and to explore the nature of the interactions. We conclude that the release of ligand (DAUDA) that accompanies binding to anionic vesicles is best explained by a phospholipid molecule interacting with the binding cavity, this event resulting in ligand displacement, rather than release of ligand being the result of major conformational changes in the vesicle-bound FABP. It is proposed that a phospholipid molecule interacts with liver FABP in an extended conformation, thus involving a single acyl chain binding at site 2.

EXPERIMENTAL

Materials

DAUDA was obtained from Molecular Probes (Junction City, OR.). Phospholipids and Br₄-DSPC were obtained from Avanti Polar Lipids (Birmingham, AL). TFE and laboratory chemicals from Aldrich-Sigma (Poole, Dorset, UK). Br₄-DSPG was kindly provided by the laboratory of Professor A.G. Lee, University of Southampton [23]. Restriction enzymes and other molecular biologicals were from Promega (Southampton, UK) and Boehringer Mannheim (Lewes, E. Sussex, UK). Mutagenic primers were provided by MWG Biotech (Ebersberg, Germany).

Molecular Biology

The preparation of a synthetic gene for rat liver FABP [24] and expression using a pET-11a vector [9] has been described. Site directed mutagenesis was performed using standard cloning procedures as described previously [25]. The oligonucleotide sequences used for the construction of the mutants (mutant sequence is underlined) were:

L28W: 5'-GGGTCTGCCGGAAGACTTGGATCCGAAAGGTAAAG-3'

M74W: 5'-TGCGAACTCGAGACCTTGGACCGGTGAAAAAAG-3'

The mutated liver FABP construct was verified by sequence analysis that was carried out by Oswel (Southampton, UK).

Protein Expression and Measurements

The expression and purification of the L28W mutant protein has been described previously [22] and the M74W mutant protein was prepared by the same method. Both proteins were delipidated using Lipidex-1000 [26].

Unless stated, all ligand and phosphate binding assays were performed in 10mM HEPES/NaOH buffer pH 7.5 [22]. Phospholipid vesicles were produced by injection of the phospholipid (1mg/ml in methanol) directly into the assay cuvette. The effects of ligands or DOPG vesicles on Trp-28 fluorescence were monitored using a Hitachi F2000 fluorimeter with excitation at 285nm and emission at 340nm. Loss of DAUDA fluorescence was monitored at 500nm after excitation at 350nm as described previously [9;10]. Fluorescence emission spectra were recorded on a Hitachi F2500 fluorimeter.

CD measurements were performed using a Jasco J-270 spectropolarimeter in 10mM phosphate buffer, pH 7.4. Spectra were measured between 190 and 250 nm.

RESULTS

Phospholipid vesicle binding properties of L28W and M74W

We have recently reported the effectiveness of the tryptophan insertion mutant protein, L28W, within the portal region for reporting ligand binding to liver FABP [22] where large changes in fluorescence intensity are observed. The sensitivity of L28W to lipid interaction suggested that this protein would be effective in directly demonstrating the interaction of liver FABP with anionic phospholipid vesicles and allowing a more detailed examination of the phenomenon. Moreover we have observed that the fluorescence properties of another portal mutant protein, M74W, were also sensitive to interfacial binding even though the fluorescence intensity of this protein was minimally affected on binding fatty acids.

The binding of the L28W mutant protein to DOPG vesicle resulted in a large increase in fluorescence intensity and a significant 'blue shift' in wavelength maximum to 337.5 nm compared with 341.0 nm for the apo-protein (Table 1). The increase in fluorescence intensity indicated a major perturbation of the protein in the region of position 28 similar to that seen for fatty acid and bile acid binding [22]. The same increase in signal intensity with L28W was also observed when using 20 mol% DOPG in DOPC but no signal change was seen with pure DOPC vesicles (data not shown), consistent with the lack of ability of the protein to bind to zwitterionic vesicles as an initial electrostatic complex [9].

The titration of DOPG vesicles into a fixed concentration of L28W was monitored by the increase in fluorescence intensity (Figure 2A) and showed a saturation curve that paralleled the loss of fluorescence when the fluorescent fatty acid, DAUDA, was released from liver FABP on addition of DOPG vesicles (Figure 2A) [9]. Both phenomena are also plotted as percentage of maximum fluorescence change (Figure 2B). Thus DAUDA release monitored as a fall in fluorescence intensity and the fluorescence enhancement for the L28W mutant protein on binding to anionic phospholipid vesicles appear to reflect the same overall molecular events. Moreover, the concentration of DOPG required to produce 50% of the maximum increase in fluorescence for L28W was the same as that required to reduce DAUDA binding by 50% (data not shown). We have previously provided direct evidence using gel filtration that the protein binds to the anionic phospholipid vesicles [9].

In the case of DAUDA release [9] the phenomenon was sensitive to the ionic strength of the medium consistent with initial non-specific electrostatic interactions involving Lys-31, Lys-36 and Lys-57 [10], and this was also the case for the increase in fluorescence intensity of L28W. The binding of L28W to DOPG vesicles was prevented in the presence of high salt while the addition of NaCl after the addition of DOPG resulted in only partial loss of fluorescence (data not shown). This result is consistent with previous studies [9] and indicates a hydrophobic component to membrane binding subsequent to initial electrostatic interactions. Overall, the data is consistent with the minimal model:



FABP:DOPG* is an initial interfacial complex resulting from electrostatic interactions while FABP:DOPG** is a more stable complex involving non-polar interactions between the protein and the anionic phospholipid bilayer. High salt will prevent the formation of FABP:DOPG* but has only a modest effect on reversal of the presumptive non-polar interactions seen in FABP:DOPG**.

In contrast to L28W, the fluorescence intensity of the M74W mutant protein was significantly decreased in the presence of DOPG vesicles (Table 1). Surprisingly, the binding of oleic acid to M74W (Table 1) resulted only in a small reduction in overall fluorescence. In addition to fatty acids, liver FABP binds both lysophospholipids and certain bile salts such as those derived from lithocholic acid [27] at a presumed 1:1 stoichiometry. The binding of lysoPG to M74W resulted in a

minimal decrease in fluorescent intensity whereas quenching was much more significant on binding the dianionic conjugated bile salt, lithocholic acid 3-sulphate (Table 1). Thus the two portal mutant proteins L28W and M74W demonstrate different fluorescent characteristics when binding to ligands and DOPG vesicles.

Effect of brominated phospholipids on the fluorescence properties of L28W and M74W

Brominated phospholipids in which bromine atoms are introduced into the acyl chains have been used as effective fluorescence quenching agents [23;28;29]. Brominated phospholipids have been shown not to significantly perturb the bilayer [30]. The mechanism of quenching of tryptophan fluorescence by bromine is not clear, as it could be either a collisional event or the result of fluorescence energy transfer [28;31]. Either way, the quenching will be very sensitive to the distance between brominated ligand and the tryptophan.

The results of titrating Br₄-DSPG vesicles into L28W or M74W are shown in Figure 3. It is clear that both tryptophan residues are effectively quenched by Br₄-DSPG with the tryptophan at position 28 being more susceptible to quenching than that at position 74. When Br₄-DSPC was used instead of Br₄-DSPG, as anticipated, no quenching was observed because the FABP is unable to bind to zwitterionic vesicles (Figure 3). When titrations were performed with Br₄-DSPG in the presence of 0.2M NaCl no quenching was observed (data not shown) further emphasising the importance of initial electrostatic interactions in the overall process.

The high affinity of the binding of liver FABP to the phospholipid interface was confirmed by titrating in Br₄-DSPG to saturation followed by addition of DOPG or *vice versa*. If liver FABP was able to exchange rapidly between brominated and non-brominated vesicles, corresponding changes in fluorescence intensity would have been observed. In fact no significant fluorescence change was observed on titrating in DOPG to FABP previously bound to Br₄-DSPG vesicles or on titrating in Br₄-DSPG to FABP previously bound to DOPG vesicles over the time course of the experiment. This result confirms that once the protein had bound to one set of vesicles it becomes anchored to those vesicles and does not exchange onto different vesicles subsequently added to the assay (Figure 4).

We have previously shown that it is not possible to detect DAUDA binding to vesicle-bound FABP, an observation that is not due to preferential partitioning of the DAUDA into the anionic phospholipid bilayer. Similarly the addition of oleic acid

and other ligands to the quenched protein bound to Br₄-DSPG failed to bring about any recovery in fluorescence consistent with high affinity interactions between the brominated acyl chains of the phospholipid and the vesicle-bound protein (data not shown).

The CD spectrum of L28W after interacting with ligands, phospholipids or TFE

In order to gain insight into the conformational state of the liver FABP when bound to DOPG vesicles, CD measurements were performed on the protein in solution and when bound to phospholipid vesicles. Figure 5 compares the effects of oleic acid, DOPC and DOPG on the CD spectrum of L28W. A very modest change in spectrum is seen in the presence of DOPG (Figure 5; spectrum D) that may be explained by increased sample turbidity due to aggregates of L28W and DOPG affecting values obtained at lower wavelengths with no change at 190nm or 220nm. This indicates that there is no major change in secondary structure on vesicle binding. No changes in the CD spectrum are seen after the addition of oleic acid or DOPC (Figure 5; spectra B and C) and this result provides further evidence of the interaction of liver FABP with DOPG vesicles compared with DOPC vesicles while fatty acid binding does not significantly affect protein structure, as originally shown in the case of intestinal FABP [32].

In order to provide a positive control for changes in CD, the protein was dissolved in 50% TFE following conditions used for the related protein, CRABP [33]. There was a large enhancement of α -helical content of liver FABP (Figure 5; spectrum E) characterised by the increase in ellipticity at 220nm and 190nm. This major change in protein structure, also seen with CRABP [33] and β -lactoglobulin [34], is highly cooperative which suggests that the protein may reversibly flip between two conformations at a critical concentration of TFE (~40%v/v; data not shown).

DISCUSSION

Previously, we have highlighted the effectiveness of the L28W portal mutant protein to monitor ligand binding, due in particular to the dramatic increase in fluorescence intensity when this mutant protein binds fatty acids [22]. In this paper we have used L28W and also M74W portal mutant proteins to examine the effect of the binding of liver FABP to anionic phospholipid vesicles containing DOPG, and have used the

fluorescence quenching ability of brominated phospholipids to further dissect the interactions.

When the L28W mutant protein bound to DOPG vesicles a large increase in fluorescence intensity was seen that was accompanied by a significant blue shift in the wavelength of maximum fluorescence emission (Figure 2; Table 1). The increase in tryptophan fluorescence intensity provided us with a means to directly monitor binding of liver FABP to the phospholipid interface. Previously we utilised the large decrease in fluorescence that resulted from the release of the fluorescent ligand DAUDA from a liver FABP:DAUDA complex to monitor indirectly the phenomenon [9;10].

The binding of L28W to DOPG vesicles was sensitive to the ionic strength of the medium and the initial binding could be eliminated at high salt concentrations consistent with the formation of an initial electrostatic complex at the phospholipid interface (FABP:DOPG*). However reversal of interfacial binding was not readily reversed by subsequent addition of high salt indicating further restructuring involving hydrophobic interactions to produce FABP:DOPG**. We had previously noted similar phenomena when studying wild type FABP binding to anionic phospholipids by monitoring DAUDA release [9].

The precise nature of the FABP:DOPG** complex is not known but should have some similarities to that observed when ligands bind producing enhanced fluorescence with L28W although in the case of DOPG binding a small “blue shift” in emission maximum is also observed (Table 1). In the case of fatty acid binding to L28W we have argued that the dramatic increase in fluorescence is due to the release from quenching of Trp-28 by an adjacent group in the protein due to the presence of the alkyl chain of the fatty acid [22]. Presumably in the presence of DOPG vesicles, Trp-28 also experiences a non-quenching environment consistent with the insertion of an acyl chain into the binding cavity of the protein.

The considerable fluorescence quenching observed when M74W binds to DOPG vesicles is not mirrored by ligand binding. The fluorescence quenching is minimal in the case of fatty acids and lysophospholipids (Table 1). In contrast significant quenching is seen with binding of the bile acid derivative lithocholic acid 3-sulphate indicating that the tryptophan at position 74 is sensitive to the nature of the bound ligand. Overall it would appear that binding to an anionic phospholipid vesicle produces a more quenching environment at position 74 of the portal region than is experienced when ligands bind.

Quenching studies involving brominated phospholipid (Br₄-DSPG) where bromines are inserted at positions 9 and 10 of the acyl chain produced apparent quenching of 89% and 77% of the tryptophan for L28W and M74W respectively (Figure 3). This high level of quenching requires that a bromine is less than 8 Å from tryptophans at positions 28 or 74 because a separation distance of 8 Å is that required to give 50% quenching [23;28;29]. Since quenching is a function of the sixth-power of distance (R^6) it is a very sensitive measure of proximity. This distance constraint is consistent with an acyl chain from the phospholipid occupying a position equivalent to site 2 where it can be calculated that bromines attached to C9 or C10 of oleic acid are about 6 Å from the C α carbon at both positions 28 and 74 (Figure 1). This is not the case for a brominated oleic acid located at site 1 where distances are about 20 Å for position 28 and 13 Å for position 74. Moreover, binding of the FABP to the surface of the bilayer without major perturbation of the bilayer would position the bromines of Br₄-DSPG within the hydrophobic core of the bilayer at least 20 Å from the membrane surface, a distance that would result in minimal quenching.

A model to illustrate the possible interaction between liver FABP and DOPG vesicles is shown in Figure 6A. The initial orientation of liver FABP with respect to the phospholipid vesicle surface locates Lys-31, Lys-36 and Lys-57 at the anionic phospholipid interface [10] and positions the protein to allow access of a phospholipid molecule to the binding cavity. Phospholipid binding would result in the displacement of a ligand such as DAUDA previously observed, while CD analysis indicates only very minor changes in protein structure when the protein interacts with anionic vesicles suggesting the binding cavity is preserved.

What is the nature of the interaction of a phospholipid molecule with liver FABP? In the case of cytochrome c binding to anionic phospholipid vesicles it is proposed that a phospholipid molecule adopts an extended conformation with one acyl chain extending out of the bilayer and interacting directly with the protein [16], thus anchoring the protein to the bilayer in a process involving hydrophobic interactions. It is argued that with cytochrome c, a fatty acid binding site in this protein can accommodate a fatty acid of the phospholipid because the spectral changes observed when the protein binds to liposomes containing a high mole fraction of DOPG were identical to those observed for the binding of oleic acid [16].

The results reported above support such a mechanism in the case of liver FABP as illustrated in Figure 6B. Thus an acyl chain would be accommodated at site 2 producing the expected fluorescence enhancement seen using the L28W mutant

protein [22] while the considerable fluorescence quenching produced using brominated phospholipids is consistent with a brominated fatty acid acyl chain being located at site 2 in close proximity to Trp-28. Similarly, such a brominated acyl chain would be in close proximity of Trp-74. The insertion of an acyl chain into the ligand binding cavity would explain the DAUDA displacement previously observed when a liver FABP:DAUDA complex binds to anionic vesicles. An alternative theoretical possibility is that the liver FABP could accommodate both acyl chains of a phospholipid molecule. This is unlikely because a molecule of DOPG has a molecular volume of $\sim 700 \text{ \AA}^3$ [35] compared with a value of 610 \AA^3 for the central cavity of liver FABP [36] while it is not clear how complete binding of a phospholipid molecule to liver FABP would enhance the anchoring of the protein to the phospholipid interface to produce a stable FABP:DOPG** complex.

In summary, the binding of liver FABP to small anionic phospholipid vesicles results in ligand release. No major changes in protein conformation are observed under these conditions and it is argued that the stable complex of protein with phospholipid interface allows an acyl chain from an extended phospholipid molecule to interact with the ligand-binding cavity of the protein via the portal region thus resulting in the release of any bound ligand. An extended phospholipid mechanism of membrane anchorage may have more general applicability for protein-membrane interactions and has been discussed [16].

ACKNOWLEDGEMENT

We thank Professor A.G. Lee for reading the manuscript and making many helpful suggestions.

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TABLE I Effect of ligands on the fluorescent properties of the L28W and M74W mutant proteins of liver FABP

The ligand in methanol was added to the L28W mutant protein (0.8 μ M) and M74W (0.7 μ M) mutant protein of liver FABP in 10mM Hepes pH 7.5 as described under Experimental Procedures. The wavelength of maximum fluorescence and fluorescence intensity were recorded. The fluorescence intensity is normalised to 100% for the L28W and M74W in the absence of ligand. All values are the mean of measurements performed in triplicate \pm S.D.

Liver FABP mutant	Ligand Added	Ligand Concentration (μ M)	Wavelength Maximum (nm)	Maximum fluorescence intensity (% apoprotein)
L28W	None	---	341.0 \pm 0.5	100
L28W	Oleic Acid ^a	4	342.5 \pm 0.5	390 \pm 3
L28W	Oleic Acid ^a	20	342.0 \pm 0.5	405 \pm 3
L28W	DOPG (vesicles)	25	337.5 \pm 0.5	242 \pm 8
L28W	Lyso PG	5	343.0 \pm 1.0	295 \pm 5
L28W	Lithocholic acid 3-sulphate ^a	4	341.5 \pm 0.5	356 \pm 12
M74W	None	---	340.3 \pm 0.7	100
M74W	Oleic Acid	5	---	95.5 \pm 3.7
M74W	DOPG (vesicles)	25	---	27.3 \pm 0.9
M74W	LysoPG	5	---	96.9 \pm 1.0
M74W	Lithocholic acid 3-sulphate	5	---	60.5 \pm 1.5

^aData taken from [22]

FIGURE LEGENDS

Figure 1 Ribbon diagram of liver FABP showing the position of bound oleic acid (sites 1 and 2) and the position of Leu-28 and Met-74

The position of oleic acid at site 2 is shown in CPK representation while carbons 9 and 10 to which bromine atoms would be attached in brominated phospholipids are highlighted in black. Leu-28 and Met-74 are shown in ball and stick representation whereas the oleic acid at site 1 is in stick representation. The position of the carboxyl groups of the two oleic acid molecules is indicated. The protein is orientated to most clearly show the portal region and associated residues. The model is derived from the crystal structure of liver FABP [37].

Figure 2 Effect on fluorescence intensity of the addition of DOPG to either the fluorescent complex of the L28W mutant protein of liver FABP and DAUDA or to the L28W mutant protein in the absence of ligand

(A) The % change in initial fluorescence intensity after the addition of DOPG vesicles to liver FABP (0.8nmol) in 10mM Hepes buffer pH 7.5 (1ml). L28W liver FABP complexed with DAUDA (1 μ M) and monitoring loss of DAUDA fluorescence, ●; L28W liver FABP without ligand and monitoring increase in tryptophan fluorescence, ▲. (B) The change in fluorescence intensity plotted as a % of maximum change in fluorescence after addition of DOPG vesicles. DAUDA fluorescence, ●; tryptophan fluorescence, ▲. All values are the mean of titrations performed in triplicate \pm S.D..

Figure 3 Effect of the addition of brominated phospholipids on the fluorescence properties of L28W and M74W

To 0.8 nmole of L28W or M74W in 10mM Hepes pH 7.5 (1ml) was added up to 60 nmole of Br₄-DSPG and the change in fluorescence intensity as % of initial fluorescence was determined. This value is then corrected to be expressed as a % of the change that would have been observed compared with the titration using DOPG.

Control titrations involving addition of DOPC and Br₄-DSPC showed minimal changes in fluorescence intensity. L28W + Br₄-DSPG, ●; M74W + Br₄-DSPG, ■; L28W + Br₄-DSPC, ▼; M74W + Br₄-DSPC, ▲. Values are the mean of triplicate titrations ± S.D.

Figure 4 Effect of the sequential addition of DOPG and Br₄-DSPG vesicles on the fluorescence intensity of the L28W mutant protein of liver FABP

To 0.8 nmole of L28W in 10mM Hepes pH 7.5 (1ml) was added up to 30 nmole of DOPG or Br₄-DSPG followed immediately by a further addition of 30 nmole of the other phospholipid. DOPG followed by Br₄-DSPG, ●; Br₄-DSPG followed by DOPG, ○. Values are the mean of triplicate titrations ± S.D.

Figure 5 CD spectra of the L28W mutant protein of liver FABP in the absence or presence of phospholipid vesicles or ligand

All spectra were for 12.5μM L28W in 10mM potassium phosphate buffer, pH 7.4. **A**, sample contained 5% methanol; **B**, sample contained 25μM oleic acid in 5% methanol; **C**, sample contained 625μM DOPC in 5% methanol; **D**, sample contained 625μM DOPG in 5% methanol; **E**, sample contained 50% TFE.

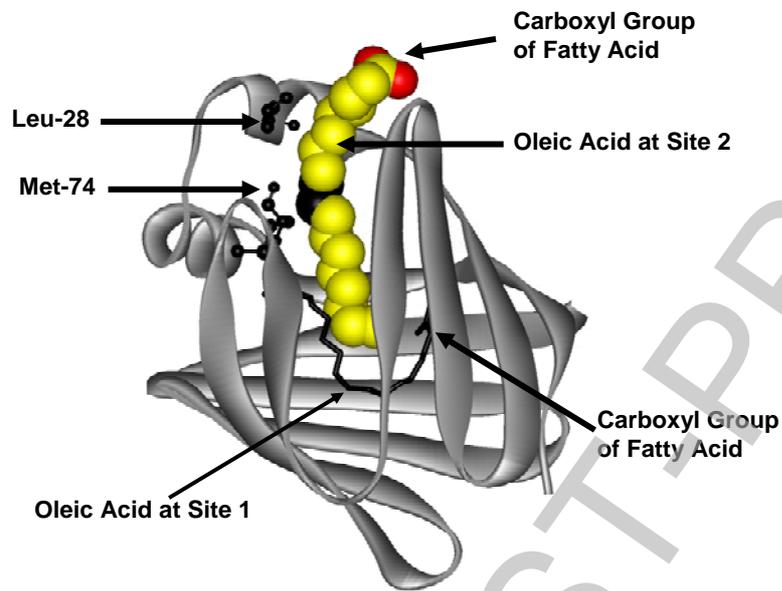
Figure 6 Model to illustrate the possible interaction of liver FABP with an anionic phospholipid interface

(A) Initial binding of liver FABP to DOPG vesicles via electrostatic interactions involves Lys-31, Lys-36 and Lys-57 (see Figure 7 in [10]). The position of Leu-28 is shown in *ball-and-stick* representation as is oleic acid bound at site 1 and site 2 of liver FABP [37]. The initial interaction with the vesicle surface is referred to as FABP:DOPG*. High affinity binding of liver FABP to anionic phospholipid vesicles will occur in the absence of ligands [9]. The FABP:DOPG** complex is predicted to involve some phospholipid penetration through the portal region either involving one (extended phospholipid conformation) or possibly two acyl chains. The model is

adapted from the crystal structure of liver FABP with two molecules of bound oleic acid [37]. **(B)** FABP:DOPG** complex showing an extended phospholipid conformation which would anchor the FABP to the bilayer.

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FIGURE 1

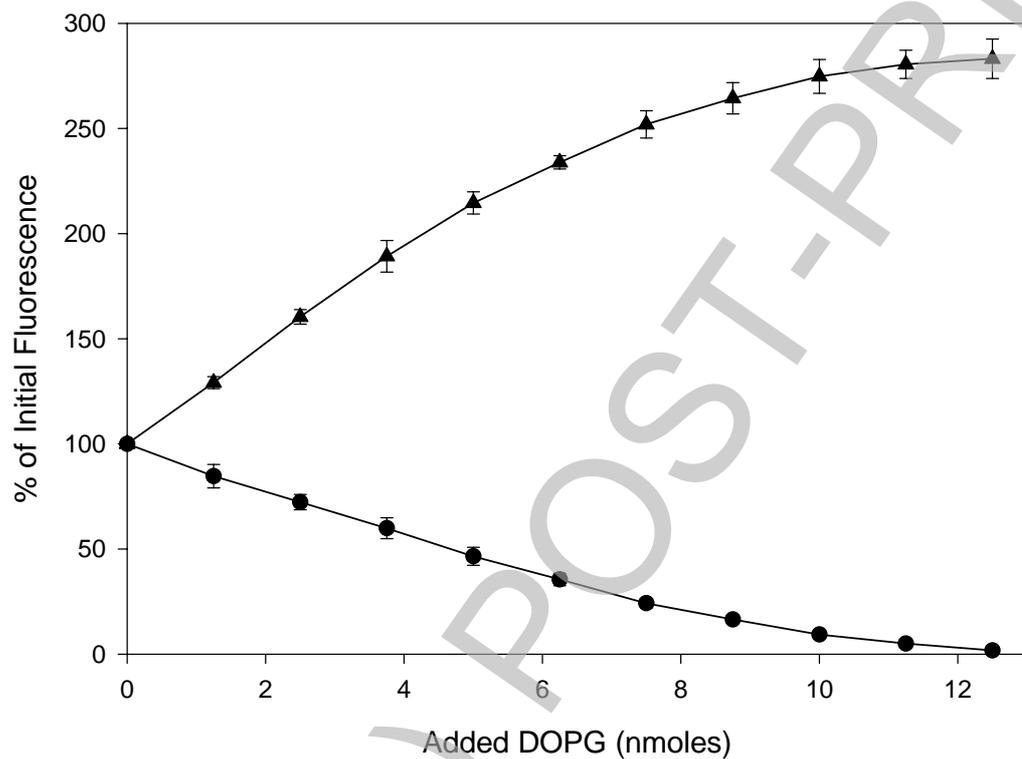


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FIGURE 2

A



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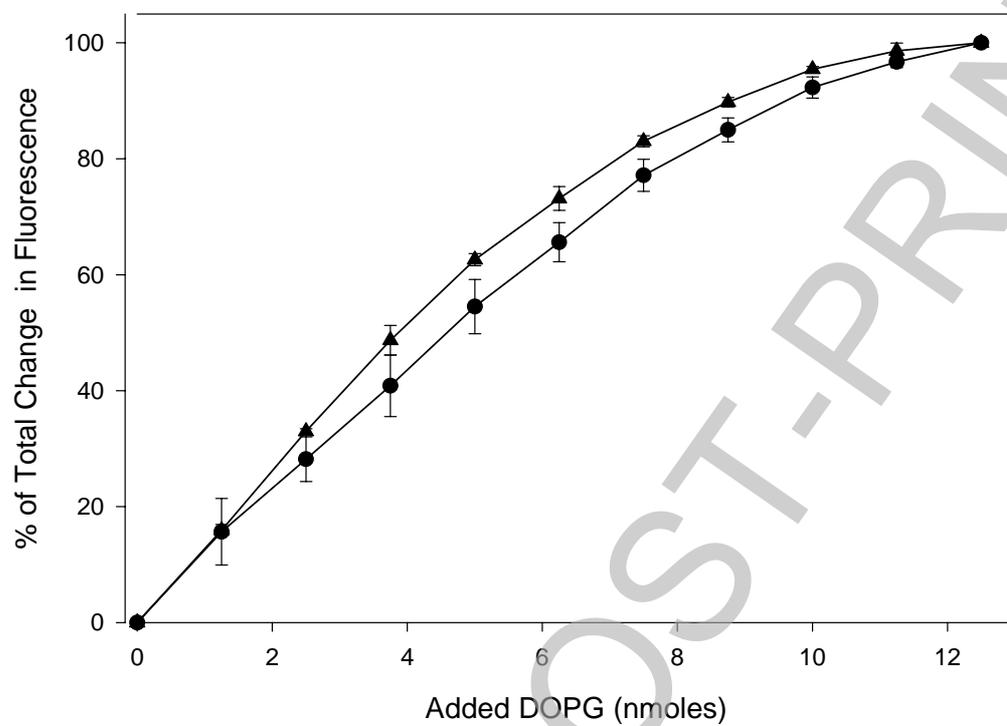
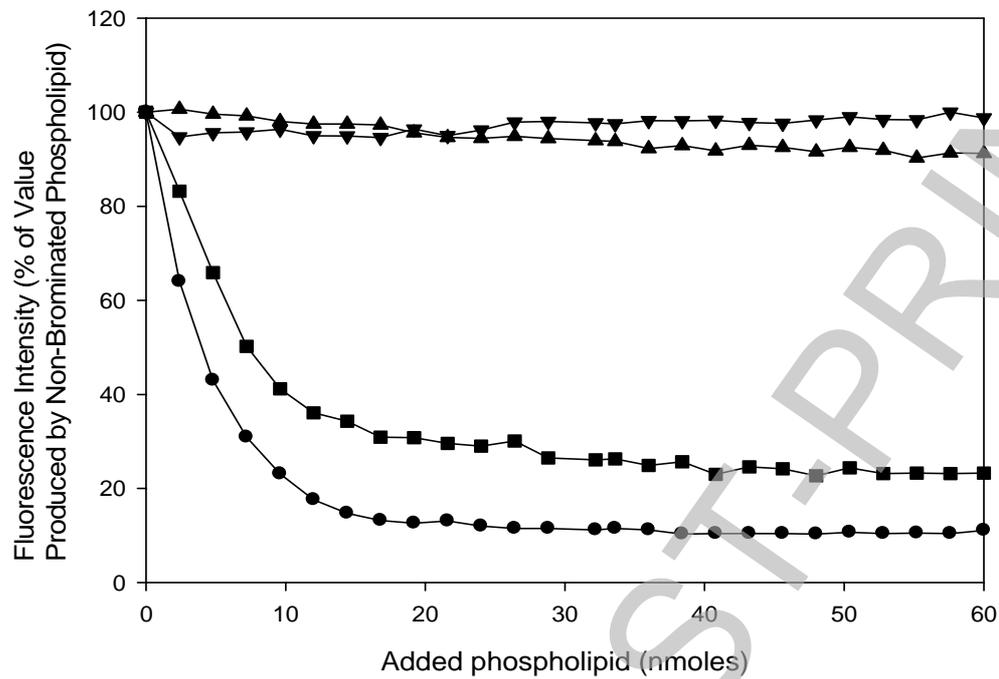
B

FIGURE 3



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FIGURE 4

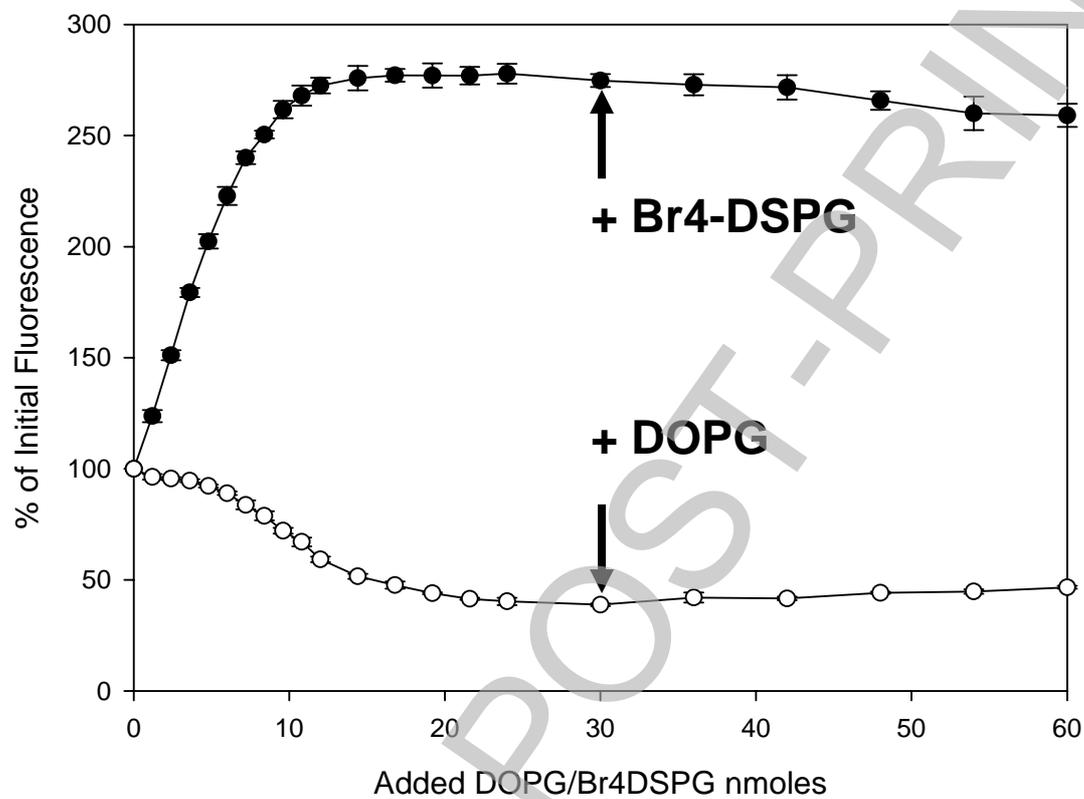
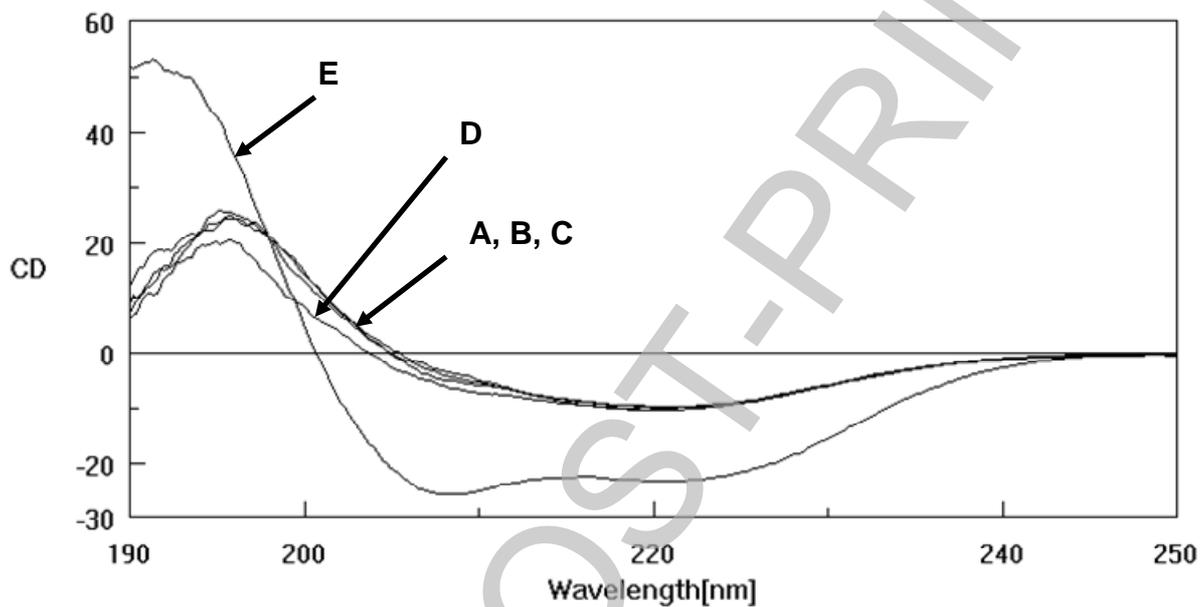


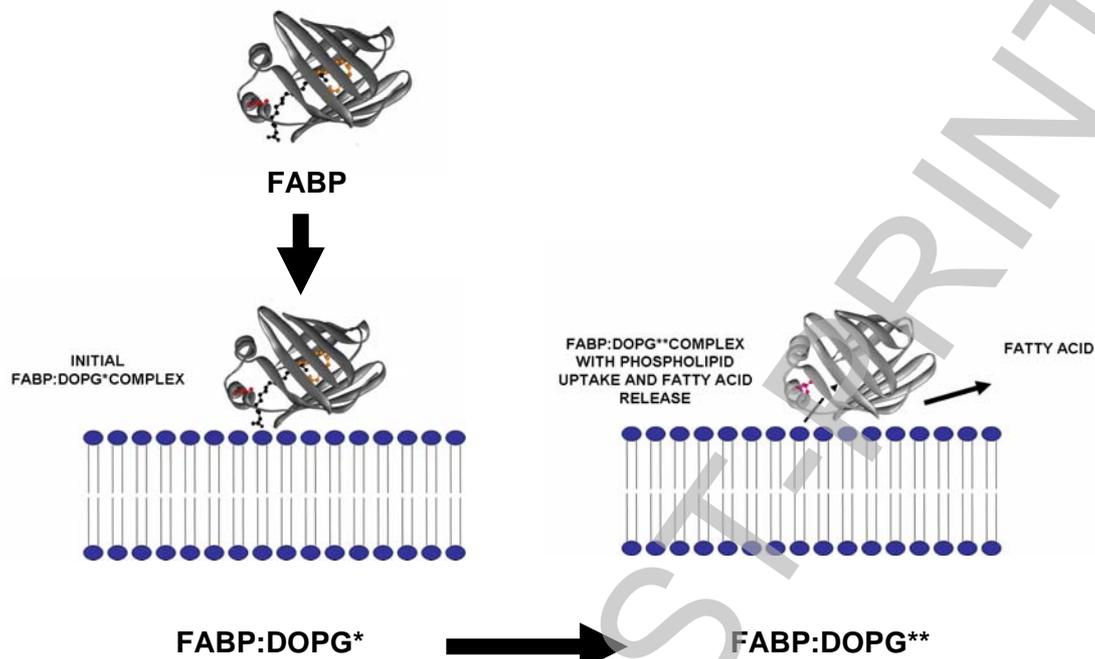
FIGURE 5



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FIGURE 6A



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FIGURE 6B

FABP:DOPG COMPLEX SHOWN AS AN EXTENDED
PHOSPHOLIPID ANCHORAGE MODEL**